Quantitation of circulating fibrinogen breakdown products in intravascular clotting

Reprint from

Diffuse Intravascular Clotting

Transactions of the Conference Held under the
Auspices of the International Committee on Haemostasis and Thrombosis
St. Moritz, Switzerland, September 1965

K. M. BRINKHOUS
Chairman

I. S. WRIGHT
Secretary General

F. KOLLER
Editor

F. STREULI
Editor

F. DUCKERT
Associate Editor

172 Figures, 51 Tables

19 66

F. K. SCHATTAUER-VERLAG · STUTTGART

18. Quantitation of Circulating Fibrinogen Breakdown Products in Intravascular Clotting

H. C. Hemker, N. Fekkes, A. Hensen, H. Schrijver and E. A. Loeliger

The case which prompted the investigation reported here concernes a 31 year old multipara who developed a peculiar defibrination syndrome after having given birth to twins who died of asphyxia during delivery.

Twenty-four hours after delivery the fibrinogen content of the plasma, estimated according to Clauss (1), appeared to be 20 mg%, but a rather firm clot formed after longer incubation with thrombin. Moreover, the patient did not bleed, and the thrombelastogram did not show any of the signs typical of a low fibrinogen content.

This case brought out strongly a discrepancy that is rather often seen and more often suspected in the laboratory, viz. the discrepancy between the fibrinogen content measured by the Clauss method and the amount of fibrin apparently formed by the same plasma.

It is known that many kinds of fibrin- and fibrinogen-breakdown products are formed during intravascular clotting. Proteolysis accompanying intravascular clotting may cause fibrinogen to lose its clottability and immunological properties. Some of the products formed may gain the ability to inhibit thrombin action, polymerization of fibrin, or stages in the prephase of blood-clotting (2—10). Fibrin may undergo a similar breakdown. For all practical purposes in the present paper this heterogeneous group of products can be divided into three sub-groups, viz.:

a) unaltered fibrinogen

b) antifibrinogen-precipitable but unclottable material

c) material inhibiting the formation rate of a fibrin clot.

We attempted to estimate the relative amounts and half-life times of the fibrin(ogen) derivatives in our patient. The antifibrinogen positive material in the serum and plasma can be estimated on the basis of immunologic reactions. Ferreira et al. (11, 12) have worked out a semi-quantitative method which was not, however, sensitive enough to suit our purposes. Schwick (13) has developed

From the Haematology Section of the Department of Internal Medicine (Head: Prof. J. Mulder, M.D.), University Hospital, Leyden, and the »Diakonesseninrichting Bronovo« (Medical Director: P. A. de Groot), The Hague, The Netherlands.

a method by which the amount of precipitate is measured nephelometrically. We adapted a method based on a principle developed by Mancini et al. (14) and also independently by Rümke (15).

In this method, the gel in which the sample diffuses, already contains the antibody, in this case rabbit anti-human fibrinogen.

The immunization scheme was suggested to us by Dr. W. Hijmans (Department of Rheumatology, University Hospital, Leyden). With an interval of 1 week, we gave 3 i.m. injections of 100 mg human fibrinogen (fraction 1—4, kindly supplied by Dr. Blombäck) with 1 ml of Freund's adjuvans. Three weeks after the last i.m. injection, increasing doses of alum-precipitated fibrinogen were given i.v. at 2- or 3-day intervals (twice 25 mg; twice 50 mg; twice 100 mg; twice 200 mg). Ten days after the last i.v. injection the rabbits were bled. The serum was incubated with one-tenth its volume of normal human serum to precipitate non-specific antibodies, centrifuged, and stored in 1 ml portions at —20°. The gel consisted of 0.8% agar in a solution containing 0.36 M NaCl, 15 mM KCl, 5 mM K-Na phosphate buffer (pH 7.4), and 1‰ sodium azide. Before solidification, 0.8% (v.v.) of anti-human fibrinogen rabbit antiserum was added, and 12 ml of the mixture poured out in a plastic Petri dish (diameter 8.5 cm). Holes with a diameter of 3 mm were punched out in the gel, and 0.01 ml of the

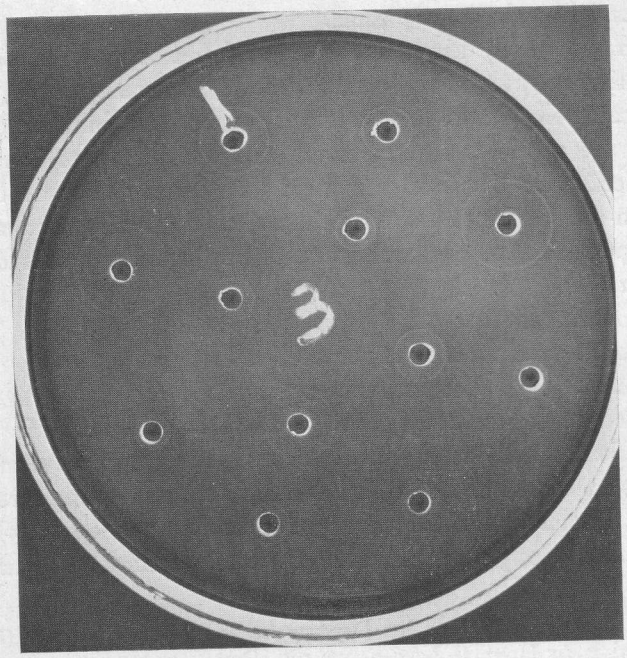

Fig. 1. A plate after incubation.

solution to be tested was pipetted into them. After 3 days incubation at 37° the dishes were photographed on a one-to-one scale, and the diameter of the precipitation rings was measured. The square of the diameter of course is proportional to the surface area.

The antigen diffuses out in the gel, and after 1—3 days of incubation at 37° a precipitation ring shows up. The area enclosed by this ring is proportional to the amount of antigen brought into the well. Fig. 1 shows a gel-plate after incubation. Fig. 2 shows the relationship between the area and the amount of antigen added with human fibrinogen as the antigen. The standard error was less than 3%.

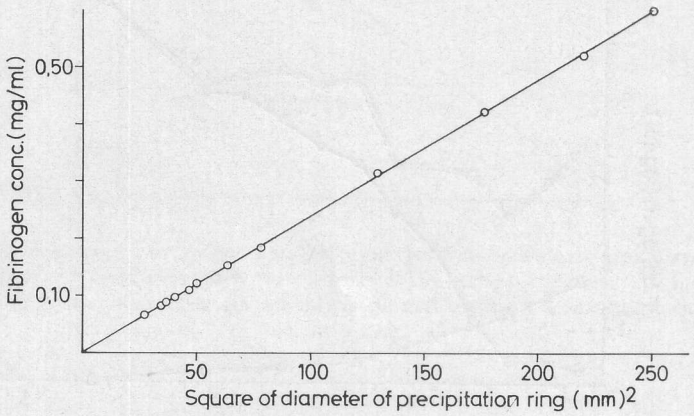

Fig. 2. The calibration curve.

It is interesting to note that with this method one precipitation ring always shows up when a mixture of antifibrinogen-positive materials is present, although it is known that fibrinogen and its breakdown products differ in their antigenic and diffusion behaviour. Because of these differences we can only express the quantity of antifibrinogen-positive material as the equivalent amount of fibrinogen giving an equal precipitation ring.

Fibrinogen itself was measured in 3 ways:

1. As the clotting of the diluted plasma with a standard amount of thrombin (Clauss method).

To 0.1 ml of an appropriate dilution (1 in 10 for normal plasma ranges) of the plasma sample in Michaelis buffer (pH 7.4), 0.1 ml of a thrombin solution (64 NIH u/ml) is added at zero time. The reaction is carried out at 37°, the moment of clotting is assessed with a Kolle hook.

2. Gravimetrically, as the amount of fibrin recovered after clotting of the plasma.

3. As the amount of antifibrinogen-positive material in the plasma minus the amount of antifibrinogen-positive material in the serum, with the assumtions that (a) no significant amount of antifibrinogen-positiv material is taken up by the clot, and (b) that this material has a similar reactivity as fibrinogen.

Of these methods, the first is sensitive to inhibiting substances, whereas the last two are not. A fairly good agreement was found between the last two methods.

Fig. 3 shows the pertinent data in our patient.

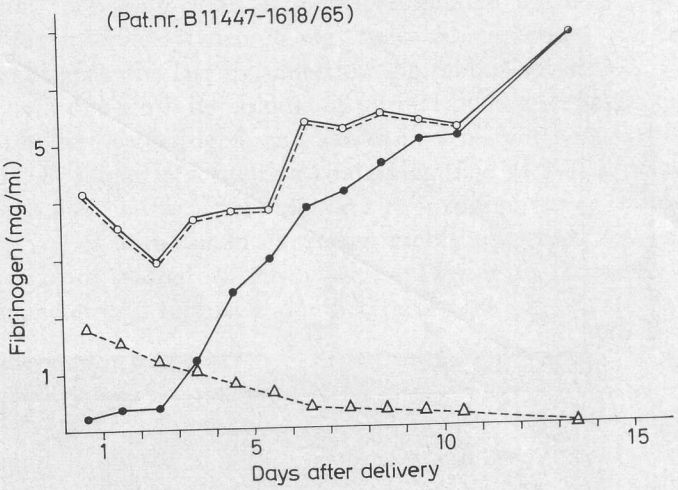

Fig. 3. The data of fibrinogen estimations obtained in the case reported. O——O Antifibrinogen-positive material in plasma, Δ - - - Δ antifibrinogen-positive material in serum (on a scale 10× the scale of the y-axis), ------ antifibrinogen-positive material in plasma minus the antifibrinogen-positive material in serum, ●——● fibrinogen content of the plasma as found with the Clauss method.

Assuming that no further intravascular proteolysis occurred after delivery, we could estimate the half-life time of the antifibrinogen-positive but unclottable material from the estimation of this material in the serum of the patient. This value proved to be 79 h, which is equal to the lower limit of the half-life of normal fibrinogen (16). It should be noted that the blood was collected in a solution containing EACA and Trasylol in order to prevent proteolysis after venipuncture. (Final concentrations: EACA 1 mg/ml, Trasylol 1 u/ml).

To estimate the half-life time of the inhibiting substances, we would first have to know how these substances act. To understand this action, we would have to know the normal kinetic behaviour of the fibrinogen-thrombin interaction; and this we do not yet know in detail. We do know, however, that a graph com-

parable to the Lineweaver-Burk plot is obtained when clotting time is plotted against the inverse of the square of the fibrinogen concentration (Fig. 5) (21).

By using this plot, the mode of inhibitory action could be assessed; it was found to be a competitive one (Fig. 6).

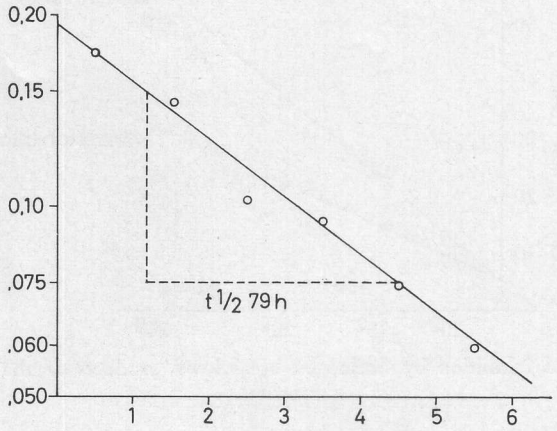

Fig. 4. The disappearance rate of the antifibrinogen positive breakdown products. The log of the concentration of antifibrinogen-positive material in serum, expressed as the equivalent concentration of normal fibrinogen (in mg/ml) is plotted on the y-axis against time (in days) on the x-axis.

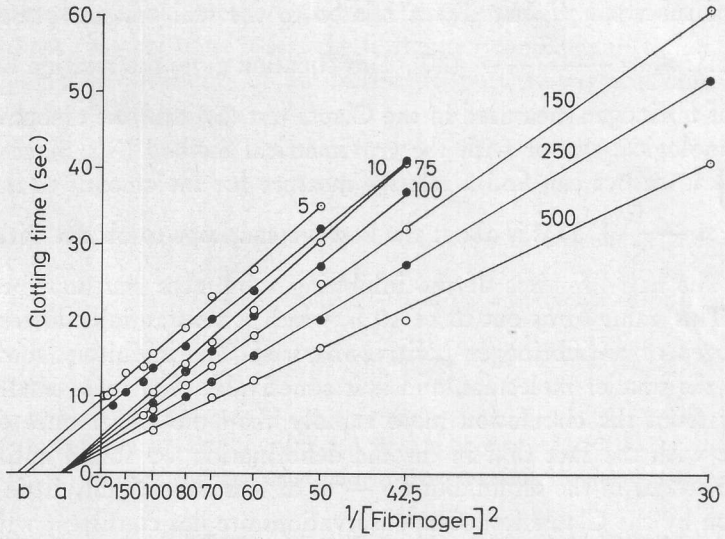

Fig. 5. The relation between fibrinogen concentration and clotting time.

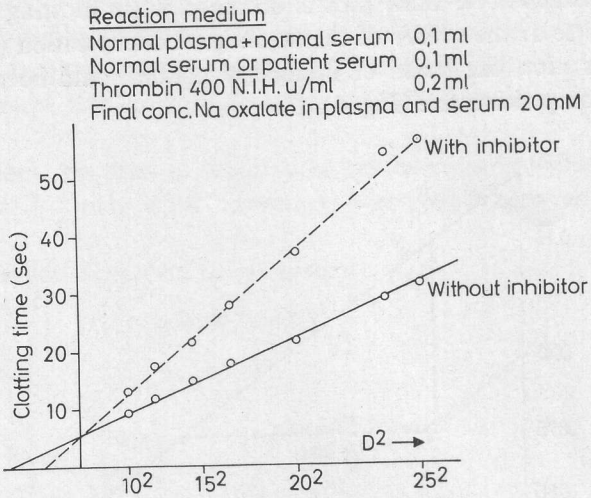

Fig. 6. The mode of inhibition by fibrinogen breakdown products of the thrombin catalysed reaction.

This finding is in agreement with the results of Triantaphyllopoulos (17) but it undoubtedly represents an oversimplification because we know that many different kinds of inhibition occur in these cases (9, 18, 19). In our experimental set-up, however, the inhibition can for all practical purposes be described as being of a purely competitive type. In competitive inhibition the apparent amount of substrate present (S_c) is related to the real amount present (S_r) by the formula: $S_c = \frac{S_r}{1 + I/K_i}$ (20). This equation gives the relation between the amount of fibrinogen measured in the Clauss test (S_c) and the amount found by the immunological test or with the gravimetrical method (S_r). Since both these data are known, we can find a relative measure for the amount of inhibitor (I) (viz.: $I \sim 1 - \frac{S_r}{S_c}$). This is about the best we can hope to obtain with this kind of data. The half-life time of the inhibiting substances can now be estimated (Fig. 7). This value turns out to be 19 h, which is substantially lower than that of fibrinogen or antifibrinogen-positive material. The inhibitory substances are probably the smaller molecules, and it is conceivable that these small molecules disappear from the circulation more rapidly than the larger ones do. This is consistent with the fact that in chronic defibrination we found antifibrinogen-positive material in the serum, but no — or at least remarkably little — inhibitory action by the Clauss test. Our observations are not consistent with the idea that the existence of a fibrinogen with altered reactivity is the main cause of the

slow fibrinogen conversion (17), because altered fibrinogen would behave as non-competitively — or incompetitively — inhibited normal fibrinogen and would have about the same half-life as normal fibrinogen has. Altered fibrinogen may, of course, be present but it is not detectable in our tests.

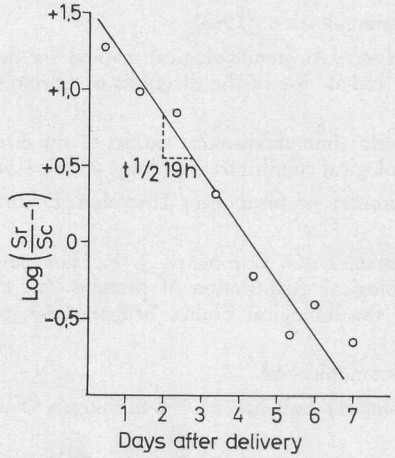

Fig. 7. The disappearance rate of thrombin inhibiting breakdown products (see text).

In conclusion, it may be said that the two-dimensional agar diffusion methods are valuable for the investigation of the defibrination syndrome, because, together with the Clauss method and the gravimetrical method of fibrinogen estimation, they enable us to assess the fibrinogen content and to get an impression of the relative amounts of antifibrinogen-positive material and thrombin-inhibiting breakdown products.

References

(1) Clauss, A.: Gerinnungsphysiologische Schnellmethode zur Bestimmung des Fibrinogens. Acta haemat. (Basel) *17:* 237 (1957).

(2) Seligmann, M.: Possibilité d'étude immunochimique de la fibrinolyse. Rev. franç. Etud. clin. biol. *3:* 1073 (1958).

(3) Salmon, J.: Etude immunochimique du fibrinogène et de ses dérivés. Clin. chim. Acta *4:* 767 (1959).

(4) Niewiarowski, S., E. Kowalski: Formation of an antithrombin-like anticoagulant during proteolysis of fibrinogen. Bull. Acad. Polon. Sc. Ser. Sc. Biol. *5:* 169 (1957).

(5) Bang, N. U.: Normal and abnormal fibrin polymerisation. Thrombos. Diathes. haemorrh. Suppl. *13:* 131 (1963).

(6) Nussenzweig, V., M. Seligmann: Dégradation par la plasmine du fibrinogène humain. Rev. Hémat. *15:* 451 (1960).

(7) Nussenzweig, V., M. Seligmann: Les produits de dégradation du fibrinogène. Ann. Inst. Pasteur *100:* 490 (1961).

(8) Niewiarowski, S., E. Kowalski: Un nouvel anticoagulant dérivé du fibrinogène. Rev. Hémat. *13:* 320 (1958).

(9) Kowalski, E., A. Z. Budzinski, M. Kopec, Z. S. Latallo, B. Lipinski, Z. Wegrzynowicz: Fibrinogen Spaltprodukt und Hämostase. Z. ges. inn. Med. *20:* 296 (1965).

(10) Marder, V.: Personal communication. (1965).

(11) Ferreira, H. C., L. G. Murat: An immunological method for demonstrating fibrin degradation. Products in serum and its use in the diagnosis of fibrinolytic states. Brit. J. Haemat. *9:* 299 (1963).

(12) Ferri, R. G., H. C. Ferreira: Immunochemical studies of the circulating products of fibrinogenolysis in certain pathological conditions. Vox sang. *8:* 356 (1963).

(13) Schwick, G.: Immunochemistry of fibrinogen. Thrombos. Diathes. haemorrh. Suppl. *13:* 85 (1963).

(14) Mancini, S., J. P. Vaerman, A. O. Carbonara, J. F. Heremans: A single radial diffusion method for the immunological quantitation of proteins. In: H. Peeters (Ed.) Proc. XIth Colloquium Protides of the Biological Fluids, Brugge, 1963, p. 370. Elsevier, Amsterdam 1964.

(15) Rümke, Ph.: Personal communication.

(16) Hart, H. Ch.: The biological half-life of ^{131}I-fibrinogen. Thrombos. Diathes. haemorrh. Suppl. *17:* 121 (1965).

(17) Triantaphyllopoulos, D. C.: Nature of the thrombin-inhibiting effect of incubated fibrinogen. Amer. J. Physiol. *197:* 575 (1959).

(18) Sharp, A. A., B. Howie, R. Biggs, D. T. Methuen: Defibrination syndrome in pregnancy. Value of various diagnostic tests. Lancet *II:* 1309 (1958).

(19) Vermylen, C., M. Verstraete: Différentiation des inhibiteurs de la conversion du fibrinogène en fibrine. Nouv. Rev. franç. Hémat. *5:* 376 (1965).

(20) Dixon, M., E. C. Webb: Enzymes. Second edition. London. Longmans, Green and Co. (1965).

(21) Hemker, H. C., P. W. Hemker, E. A. Loeliger: Kinetic aspects of the interaction of blood clotting enzymes. Thrombos. Diathes. haemorrh. (Stuttg.) *13:* 155 (1965).